(54) Title: SIMULTANEOUS ASSAY METHOD USING LANTHANIDE CHELATES AS THE LUMINOPHORE FOR MULTIPLE LABELS

(57) Abstract

An electrochemiluminescent (ECL) detection method for simultaneous assays. The method employs an excitation process whereby one or more luminophores are excited through an antenna mechanism using an oxidative or reductive coreactant. The luminophores can be lanthanide chelates and a persulfate system is used to generate ECL. The ECL emissions may be separated either by measuring at different emission wavelengths or by electrode potentials.

SIMULTANEOUS ASSAY METHOD USING LANTHANIDE
CHELATES AS THE LUMINOPHORE FOR MULTIPLE LABELS

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BACKGROUND OF THE INVENTION

Field of the Invention

10   The present invention relates to electrochemiluminescence (ECL) detection methods.
More particularly the invention has to do with a system using multiple ECL labels for
simultaneous assays.

Description of Related Art

15   In recent years there has been a great deal of interest in the use of lanthanides in
fluoroimmunoassays. This is due to the intense, delayed emission of the lanthanides which
allows the development of time-resolved fluorometric assays which are competitive with
radioimunoassays. Soini, E.; Hemmilä, *Clin. Chem.* 1979, 25, 353. Since efficient
population of the lanthanide excited state cannot be achieved via direct excitation, excited
20   state generation typically is accomplished via sensitization from a ligan excited state.
Emission intensity is also dependent upon the extent of non-radiative deactivation of the
lanthanide excited state by solvent interactions. Therefore, the more luminescent lanthanide
complexes are composed of heteroaromatic ligands which encapsulate the metal. The
photophysical properties of encapsulated lanthanides have recently been reviewed.
25 Sabbatini, N.; Guardigli, M.; Lehn. J.-M., *Coord. Chem. Rev.* 1993, 123, 201.

There is very little mention in the scientific literature of lanthanide chelates used as
luminophores in ECL processes. The lanthanides are not readily excited by ECL processes.
This accounts for the relative silence in the literature, not because people are unaware of the
materials. Lanthanides are very common materials used in television displays and time
30 resolved fluorescence techniques.

Volger and Kunkely (ACS Symposium Series 333, pp 155-168, 1987) describe an
ECL process using Tb(III)(TTFA)$_3$(o-phen), Tb(III)(TTFA)$_4$-, and Eu(III)(TTFA)$_3$(o-phen)
where TTFA = thenoyltrifluoroacetonate and o-phen = 1,10 phenanthroline. Their ECL

process used oscillating electrode voltages as the means for excitation. Weak emissions were observed. Our process utilizes a different means of excitation which produces much better emission intensities.

Hemingway, Park, and Bard (J. Am. Chem. Soc. 97, 200, 1975) describe an ECL process where the lanthanide Eu(DBM)$_3$PIP, DBM = dibenzoylmethide, PIP = piperidine, is activated by an energy transfer process from the ECL luminophore. In this case, the lanthanide is excited indirectly through formation of an exciplex.

A cathodic electroluminescence technique using lanthanides has been described by Kankare (for example see Anal. Chim. Acta 266, 205, 1992, Anal. Chim. Acta 256, 17, 1992). Cathodic electroluminescence is a different way of exciting than ECL techniques.

A common method of exciting lanthanide chelates is time resolved fluorescence; for example see I. Hemmilä, S. Dakubu, V.M. Mukkala, H. Siitari, T. Lovgren, Anal. Biochem. 137, 335-343 (1984).

An object of the invention was to develop an ECL detection method for simultaneous assays and we have developed a method which employs an excitation process for certain luminophores; particularly the lanthanides. A new excitation method for the lanthanides was needed because previous methods do not provide satisfactory intensities for simultaneous assays. Lanthanides, as luminophores, possess emission characteristics which enable the development of simultaneous assay. For example, four labels present in one reaction volume (such as in a simultaneous assay), each emission must be separately quantifiable. Using our new excitation process and lanthanides, the ECL emissions may be separated either by measuring at different emission wavelengths or by electrode potentials.

We have now discovered that ECL can be obtained from various lanthanide complexes and with different ligands. The level of the ECL signal for the Tb(2)$^{3+}$ complex is high enough to yield the lowest detection limit observed to date. We employ an "Antenna ECL" mechanism which is based upon lanthanide sensitization by another excited state, an analogous mechanism by which lanthanide emission is obtained in fluoroescence immunoassays. Additionally, this mechanism suggests that it is possible to tune the ECL peak potentials without substantially changing the emission wavelengths by modifying the ligand reduction potentials. This demonstrates that ECL could compete with the lanthanide based fluoro-immunoassays.

In summary, we have developed a new ECL process for:

(1) excitation of one or more luminophores through an "antenna" mechanism, using an oxidative or reductive coreactant; and

(2) excitation of one or more lanthanide chelates through an "antenna" mechanism, using an oxidative or reductive coreactant.

SUMMARY OF THE INVENTION

According to the invention, the ECL of lanthanides is achieved by "Antenna ECL". This process consists of the generation of a ligand excited state via the reaction of a reduced ligand with an oxidizing radical, the "electrochemical excitation" (ECX) steps. Energy transfer from the ligand excited state to the emissive state (i.e., lanthanide excited state) yields the characteristic lanthanide emission. Shown below are the reactions involved in the Antenna ECL process. In these reactions L represents the generic ligand, La represents a generic lanthanide metal, and an example of a coreactant which produces an oxidizing radical is presented, peroxydisulfate.
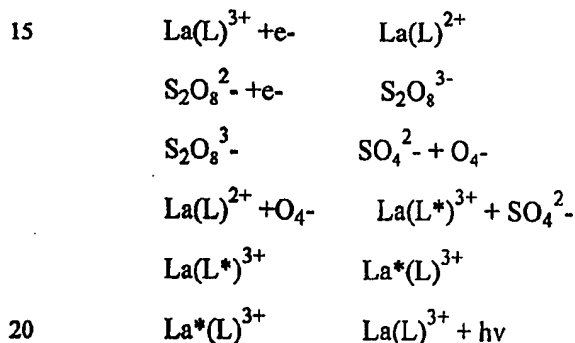
$$La(L)^{3+} + e^- \quad\quad La(L)^{2+}$$

$$S_2O_8^{2-} + e^- \quad\quad S_2O_8^{3-}$$

$$S_2O_8^{3-} \quad\quad SO_4^{2-} + O_4^-$$

$$La(L)^{2+} + O_4^- \quad\quad La(L^*)^{3+} + SO_4^{2-}$$

$$La(L^*)^{3+} \quad\quad La^*(L)^{3+}$$

$$La^*(L)^{3+} \quad\quad La(L)^{3+} + h\nu$$

wherein La represents lanthanide metal, L represents ligand, L represents a reduced ligand, L* represents a ligand excited state and La* represents a lanthanide excited state. This process has been demonstrated with five lanthanide complexes consisting of four lanthanides (Sm, Eu, Tb, and Dy) and two ligands (1 and 2), illustrated below. The complexes were $Sm(2)^{3+}$, $Eu(1)^{3+}$, $Tb(2)^{3+}$, $Tb(1)^{3+}$, and $Dy(2)^{3+}$.

LIGANDS (1 AND 2)

5

10

2   1
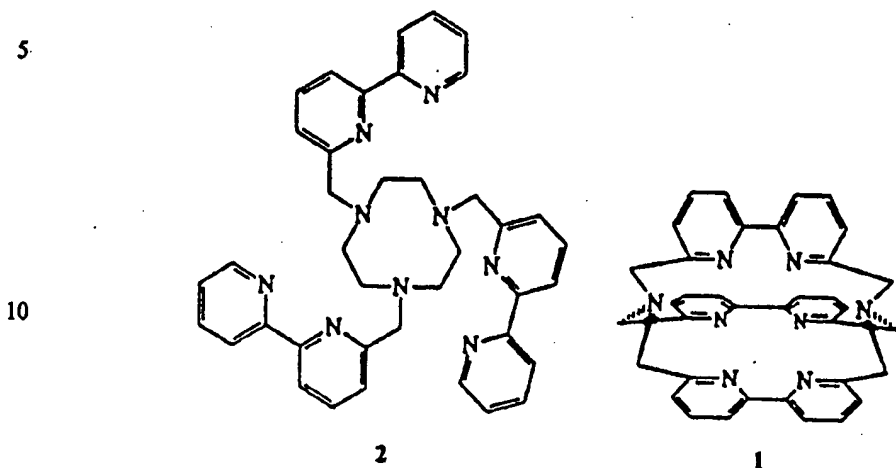
15   Each of these complexes gave ECL upon reduction of the ligand in the presence of an
      oxidizing radical.

      We have developed the ability to access lanthanide f-f excited states through
      excitation of the ligand. (For discussion of lanthanide f-f excited states see N. Sabbatini, M.
      Guardigli, J.M. Lehn; Coord. Chem. Rev. 123, 201-228 (1993), A.P.B. Sinha; Spectroscopy

20   in Inorganic Chemistry, 2, 255-288 (1971) and J.C.G. Bunzli, D. Wessner; Coord. Chem.
      Rev. 60, 191-253 (1984.) The Antenna ECL process allows access to excited states which
      cannot be accessed directly via the normal ECL processes. This feature allows the coupling
      of a species (such as the antenna or ligand) which is efficient at ECX and/or with the desired
      reduction potential (or peak potential) to another species (such as the lanthanide metal) with

25   the desired excited state properties (i.e., highly emissive, appropriate wavelength of
      emission, or non-emissive).

      We also have developed the ability to tune the potential at which ECL is observed
      through changes in the antenna (ligand) without substantially changing the emission
      wavelengths. The first step of the Antenna ECL process involves a ligand reduction.

30   Altering the ligand structure changes the reduction potential and correspondingly, the
      electrode potential for onset of ECL (excitation potential). The emission spectra is
      unchanged because it is solely related to the type a metal lanthanide present in the chelate.
      This feature is illustrated by $Tb(1)^{3+}$ and $Tb(2)^{3+}$. Using an ORIGEN® Analyzer (available

from IGEN, Inc., 16020 Industrial Drive, Gaithersburg, MD 20877 U.S.A.) the peak

potential observed for $Tb(1)^{3+}$ was ca. -3 V while that for $Tb(2)^{3+}$ was approximately

(sometimes abbreviated herein as "ca.") -5 V; however, both complexes have the

characteristic Tb^{3+} emission bands.

5

During the development of this invention, ECL from a Dy^{3+} and Sm^{3+} complex was

first observed. Additionally, we observed for the first time a lanthanide ECL with a non-

oscillating potential. This is one of three examples of aqueous reductive ECL, the other

reported examples are $Cr(phen)_3^{3+}$ (phen = 1,10-phenanthroline) by F. Bollette, M. Ciano,

10   V. Balzani, and N. Serpone, *Inorganica Chimica Acta*, 1982, *62*, 207 and $Ru(bpz)_3^{2+}$ (bpz =

2,2'-bipyrazine) by S. Yamazaki-Nishida, Y. Harma, and K. Yamashita, *Journal of

Electroanalytical Chemistry*, 1990, *283*, 455.

The lanthanide luminophores of the invention are used in a similar manner as with

$Ru(bpy)_3^{2+}$. Linker arms for attachment to various biological molecules such as antibodies

15   are used and sandwich assays for the analytes can be carried out in the usual manner.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the ligand structures 1 and 2.

Figure 2 is a cyclic voltammogram of $Dy(2)^{3+}$ in an acetonitrile solution 0.1 molar

20   ("M") in tetrabutylammonium perchlorate ("TBAP") and millimolar ("mM") in La^{3+}

recorded on platinum electrodes in volts ("V") vs. 3M Ag/AgCl.

Figure 3 is a cyclic voltammogram of $Eu(2)^{3+}$ using the same type of solution and

electrodes as Figure 2.

Figure 4 is a graph of corrected ECL vs. potential (millivolts, abbreviated "mV") for

25   aqueous $La(1)^{3+}$ using Ramp ECL (wherein the potential was continuously increased at a

rate of 4800 mV/s) with 1000 nanomolar ("nM") $Tb(1)^{3+}$ and 10,000 nM $Eu(1)^{3+}$.

Figure 5 is a graph of corrected ECL counts vs. time (centiseconds, abbreviated

"cs") for aqueous $La(1)^{3+}$ using ECL Step wherein a potential pulse was applied at -5000 mV

for 1000 milliseconds ("ms").

30   **Figure 6** is a calibration curve for aqueous $La(1)^{3+}$.

Figure 7 is a graph of corrected ECL counts vs. potential (mV) for aqueous La(2)$^{3+}$ using Ramp ECL.

Figure 8 is a graph of corrected ECL counts vs. time (cs) for aqueous La(2)$^{3+}$ using ECL Step (-5V).

5   Figure 9 is a calibration curve for aqueous La(2)$^{3+}$.

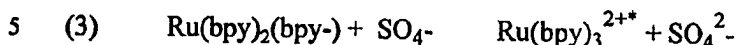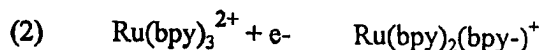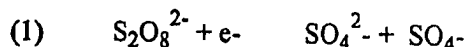## DESCRIPTION OF THE PREFERRED EMBODIMENT

The four lanthanide complexes; Tb(2)$^{3+}$, Dy(2)$^{3+}$, Sm(2)$^{3+}$, and Eu(1)$^{3+}$; can be quantified by reductive ECL in 0.1 M phosphate buffer (pH 6) which is 0.1% in surfactant

10   and 50 micromolar (" M") in potassium persulfate. A modified ORIGEN Analyzer was used for these analyses. The Analyzer was equipped with gold electrodes and a filter wheel running at ca. 10 hertz ("Hz") which had four narrow-band interference filters (613 nanometers ("nm") for Eu^{3+}, 545 nm for Tb^{3+}, 644 nm for Sm^{3+}, and 573 nm for Dy^{3+}). The filter wheel was placed between the electrode and the photomultiplier tube ("PMT") (PMT

15   model R1104 available from Hamamatsu, 360 Foothill Road, P.O. Box 6910, Bridgewater, NJ 08807 U.S.A.). A potential step of -5 V for 10 seconds ("s") was applied to generate the ECL from these complexes. Since the ECL from these complexes does not totally decay over 10 s, this long of a pulse can be utilized for integration of the signals at each wavelength. Fluorescence discrimination of these lanthanides has been described by Y.-Y.

20   Xu and I.A. Hemmilä, *Analytica Chimica Acta*, 1992, *256*, 9.

Based upon the highly luminescent properties of encapsulated lanthanides (La) and the narrowness of the emission bands (ca. 50 nm), six lanthanide complexes have been prepared with Eu^{3+}, Tb^{3+}, Dy^{3+}, Sm^{3+}, and two bipryidine based ligands, 1 and 2 (see Figure 1). The complexes prepared were, Eu(1)$^{3+}$, Eu(2)$^{3+}$, Tb(1)$^{3+}$, Tb(2)$^{3+}$, Sm(2)$^{3+}$, and Dy(2)$^{3+}$.

25   The ECL from these complexes was evaluated using a persulfate system.

The mechanism by which ECL is generated with the persulfate system is slightly different from that in the previously studied tripropylamine ("TPA") system. In the persulfate system negative potentials are applied to the electrochemical cell; thereby, reducing the label and persulfate. The reduction of persulfate involves the injection of an

30   electron into the peroxide bond which then cleaves yielding SO$_4$$^{2-}$ and SO$_4$-, a strong oxidant. The radical anion then oxidizes the reduced label yielding sulfate and excited state

label. These reactions are summarized in Equations (1), (2) and (3) below with $Ru(bpy)_3^{2+}$
(bpy = 2,2'-bipyridne) as the label.

(1) $S_2O_8^{2-} + e- \quad SO_4^{2-} + SO_4-$

(2) $Ru(bpy)_3^{2+} + e- \quad Ru(bpy)_2(bpy-)^+$

5   (3) $Ru(bpy)_2(bpy-) + SO_4- \quad Ru(bpy)_3^{2+*} + SO_4^{2-}$

EXAMPLES

All reference to water indicate deionized water. Aqueous stock solutions of $Eu(1)^{3+}$
(0.662 mM) and $Tb(1)^{3+}$ (0.986 mM) were used as received. Eight complexes with ligand 2
10   were prepared by a previously published procedure (Prodi, L.; Maestri, M.; Ziessel, R.;
Balzani, V., *Inorg. Chem,* 1991, *30,* 3798) and were used as received. These complexes
were $[Eu(2)]Cl_3$, $[Eu(2)](PF_6)_3$, $[Tb(2)]Cl_3$, $[Eu(2)](PF_6)_3$, $[Dy(2)]Cl_3$, $[Dy(2)](PF_6)_3$,
$[Sm(2)]Cl_3$, and $[Sm(2)](PF_6)_3$. Three aqueous stock solutions were prepared for each of the
chloride salts. In 5.50 milliliters ("mL") of water, 10.7 mg of $[Tb(2)]Cl_3$ was dissolved to
15   make a 2.16 millimolar ("mM") solution. A 100 microliter ("µL") aliquot was diluted to 100
mL with water to make a 2.16 micromolar ("µM") solution, a 10 mL aliquot of this was
diluted to 100 mL with water to make a 0.216 µM solution. In 6.64 mL of water, 12.0 mg of
$[Dy(2)]Cl_3$ was dissolved to make a 2.00 mM solution. A 100 µL aliquot of the 2.00 mM
solution was diluted to 100 mL with water to make a 2.00 µM solution; a 10 mL aliquot of
20   the 2.00 µM solution was diluted to 100 mL with water to make a 0.200 µM solution. In
7.02 mL of water, 12.5 mg of $[Sm(2)]Cl_3$ was dissolved to make a 2.00 mM solution. A 100
µL aliquot of the 2.00 mM solution was diluted to 100 mL with water to make a 2.00 µM
solution; 10mL of the 2.00 µM solution was diluted to 100 mL with water to make a 0.200
µM solution. In 6.50 mL of water, 11.6 mg of $[Eu(2)]Cl_3$ was dissolved to make a 2.00 mM
25   solution. A 100 µL aliquot of the 2.00 mM solution was diluted to 100 mL with water to
make a 2.00 µM solution and a 10 µL aliquot was diluted to 100 mL with water to make a
0.200 µM solution.

Photophysics

30   Ultraviolet-visible ("UV-visible") spectra were recorded with a HP 8452A Diode
Array spectrophotometer (available from Hewlett Packard Company, 2101 Gaither Road,
Rockville, MD 20850 U.S.A.). A 100 µL aliquot of each mM aqueous solutions was diluted

with 5.61 mL of water to make the following aqueous solutions: 37.8 µM Tb(2)$^{3+}$ and 35.0 µM of Eu(2)$^{3+}$, Sm(2)$^{3+}$, and Dy(2)$^{3+}$. UV-visible spectra (180-820 nm) were measured for each of the ca. 35 µM aqueous La(2)$^{3+}$ solutions.

Steady state fluorescence and quantum yield measurements were made on a Perkin-Elmer LS-5 Fluorescence spectrophotometer equipped with a Kipp and Zonen X-Y recorder model BD-91 (available from VWR Scientific, P.O. Box 626, Bridgeport, NJ 08014 U.S.A.). A scan speed of 60 nm/minute ("nm/min") was employed with the emission and excitation slits at 5 millimeters ("mm"). For quantum yield measurements, the solution absorbencies were measured with a Perkin-Elmer Lambda 4B UV-VIS spectrophotometer (available from Perkin-Elmer Corp., 761 Main Ave., Norwalk, CT 06859 U.S.A.). All solutions for the excited state measurements were air equilibrated. Aqueous solutions of the La(2)$^{3+}$ complexes were prepared such that each had an absorbance of ca. 0.10 at 320 nm. The absorbencies were 0.105 (Tb^{3+}), 0.101 (Dy^{3+}), 0.106 (Sm^{3+}), and 0.105 (Eu^{3+}). With an excitation wavelength of 320 nm the emission spectra for each complex was measured from 400 nm to 635 nm. The excitation spectra were recorded for each complex with an emission wavelength of 544 nm (Tb^{3+}), 575 nm (Dy^{3+}), 568nm (Sm^{3+}), and 620 nm (Eu^{3+}). The instrument scale used in recording the excitation and emission spectra for each complex was 0.05 (Tb^{3+}), 0.05 (Eu^{3+}), 1.00 (Dy^{3+}), and 2.00 (Sm^{3+}).

Electrochemistry

Cyclic voltammograms were recorded with a EG&G PAR 173 Potentiostat/Galvanostat (available from EG&G Princeton Applied Research, P.O. Box 2565, Princeton, NJ 08543 U.S.A.) controlled by a EG&G PARC 175 Universal Programmer (available from EG&G Princeton Applied Research, P.O. Box 2565, Princeton, NJ 08543 U.S.A.) and plotted with a Kipp and Zonen X-Y recorder model BD-91. Platinum working and counter electrodes were used and the voltages were referenced to a 3M Ag/AgCl electrode. The solutions were prepared from JT Baker reagent grade acetonitrile (available from VWR Scientific, P.O. Box 626, Bridgeport, NJ 08014 U.S.A.) stored over molecular sieves (4), which was 0.1 M in tetrabutyl ammonium perchlorate and purged with nitrogen. The cyclic voltammograms for each of the [La(2)](PF$_6$)$_3$ complexes were measured from 2.5 V to -2.3 V. The complex concentrations were 2.3 mM (Tb^{3+}), 2.1 mM (Dy^{3+}), 1.9 mM (Sm^{3+}), and 2.6 mM (Eu^{3+}).

ECL Measurements

ECL measurements were made with a modified ORIGEN Analyzer. The modifications included a flow cell with gold electrodes, a positioning plate (which centered the PMT over the working electrode), and a Hamamatsu R268 (blue sensitive PMT) (available from Hamamatsu, 360 Foothill Road, P.O. Box 6910, Bridgewater, NJ 08807 U.S.A.). A 50 µM persulfate buffer solution (see below) was used. ORIGEN cell cleaner (available from IGEN, Inc.) was used without modification. The heaters were set at 35 C and a preoperative potential ("POP") of O V was used. (POP is the initial potential at the beginning of a step or ramp.)

A 50 µM persulfate buffer solution was prepared from 10.2 mg of potassium persulfate (available from Aldrich, 1001 West Saint Paul Ave., Milwaukee, WI 53233 U.S.A.) and 750 mL of a 0.1 M phosphate buffer (pH 6.1-6.0) solution that was 0.1% in Triton X100 (available from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 U.S.A.). Aliquots of the $Eu(1)^{3+}$ aqueous stock, 151 µL and 15.1 µL, were diluted with 10 mL of the persulfate buffer to make 10 µM and 1 µM $Eu(1)^{3+}$ persulfate buffer solutions. A 100 µL aliquot of the 10 µM $Eu(1)^{3+}$ persulfate buffer solution was diluted with 10 mL of persulfate buffer to make a 100 nM $Eu(1)^{3+}$ persulfate buffer solution. Aliquots of the $Tb(1)^{3+}$ aqueous stock, 101 µL and 10.1 µL, were diluted with 10 mL of the persulfate buffer to make 10 µM and 1 µM $Tb(1)^{3+}$, persulfate buffer solutions. Aliquots, 100 µL and 10 µL, of the 10 µM $Tb(1)^{3+}$ persulfate buffer solution were diluted with 10 mL of persulfate buffer to make 100 nM and 10 nM $Tb(1)^{3+}$ persulfate buffer solutions.

The instrument program to measure ECL was used with a ramp to -5 V and a rate of 4800 mV/s. 800 V was applied to the PMT. The following solutions were analyzed: persulfate buffer, 100 nM $Eu(1)^{3+}$, 1 µM $Eu(1)^{3+}$, 10 µM $Eu(1)^{3+}$, 10 nM $Tb(1)^{3+}$, 100 nM $Tb(1)^{3+}$, 1 µM $Tb(1)^{3+}$, and 10 µM $Tb(1)^{3+}$.

Potential step analyses were performed with a step to -5 V and a pulse width of 10 s on persulfate buffer, 100 nM $Eu(1)^{3+}$, 1 µM $Eu(1)^{3+}$, 10 µM $Eu(1)^{3+}$, 10 nM $Tb(1)^{3+}$, 100 nM $Tb(1)^{3+}$, 1 µM $Tb(1)^{3+}$, and 10 µM $Tb(1)^{3+}$. As above, 800 V was applied to the PMT.

The ECL from the $La(2)^{3+}$ complexes was studied with a ramp to -5 V at a rate of 4800mV/s as well as with a step potential to -5 V for 1 s. For both types of analyses 800 V were applied to the PMT. A 50 µM persulfate buffer solution was prepared as above by dissolving solid $K_2S_2O_8$ in a 0.1 M phosphate buffer (pH 6) which was 0.1% in Triton X100. Aliquots of the three aqueous $Tb(2)^{3+}$ stock solutions; 0.216 µM (50 µL and 250 µL), 2.16

µM (50 µL, 125 µL, 250 µL, and 500 µL), and 2.16 mM (5 µL and 250 µL); were diluted with 10 mL of persulfate buffer to make the following $Tb(2)^{3+}$ - persulfate solutions: 1.08 nM, 5.4 nM, 10.8 nM, 27 nM, 108 nM, 1080 nM and 10800 nM. Three tubes of each of these solutions and three tubes of persulfate buffer were analyzed by ECL with the potential

5   ramp and potential step. Aliquots of two aqueous $Dy(2)^{3+}$ stock solutions; 2.0 µM (50 µL, 125 µL, 250 µL, and 500 µL) and 2.0 mM (5 µL and 50 µL); were diluted with 10 mL of persulfate buffer to make the following $Dy(2)^{3+}$ - persulfate solutions: 10 nM, 25 nM, 50 nM, 100 nM, 1000 nM, and 10000 nM. Three tubes of each of these solutions and three tubes of persulfate buffer were analyzed by ECL with the potential ramp and potential step.

10   Aliquots of the three aqueous $Eu(2)^{3+}$ stock solutions; 0.2 µM (50 µL and 250 µL), 2.0 µM (50 µL, 250 µL, and 500 µL), and 2.0 mM (5 µL and 50 µL); were diluted with 10 mL of persulfate buffer to make the following $Eu(2)^{3+}$ - persulfate solutions: 1.0 nM, 5.0 nM, 10 nM, 25 nM, 50 nM, 100 nM, 1000 nM, and 10000 nM. Three tubes of each of these solutions and three tubes of persulfate buffer were analyzed by ECL with the potential ramp

15   and potential step. Aliquots of two aqueous $Sm(2)^{3+}$ stock solutions; 2.0 µM (50 µL, 125 µL, 250 µL, and 500 µL) and 2.0 mM (5 µL and 50 µL); were diluted with 10 mL of persulfate buffer to make the following $Sm(2)^{3+}$ - persulfate solutions: 10 nM, 25 nM, 50 nM, 100 nM, 1000 nM, and 10000 nM. Three tubes of each of these solutions and three tubes of persulfate buffer were analyzed by ECL with the potential ramp and potential step.

20   A second set of $Sm(2)^{3+}$ - persulfate buffer solutions were prepared by diluting with 10 mL of persulfate buffer a 500 µL aliquot of the 2.0 µM aqueous solution, 5 µL and 50 µL aliquots of the 2.0 mM aqueous solution, and 250 µL and 500 µL aliquots of the 10000 nM $Sm(2)^{3+}$ - persulfate buffer solution to make 100 nM, 250 nM, 500 nM, 1000 nM, and 10000 nM $Sm(2)^{3+}$ - persulfate buffer solutions. Both sets of $Sm(2)^{3+}$ - persulfate buffer solutions

25   were analyzed in triplicate with three tubes of persulfate buffer by ECL with a potential ramp and potential step.

Photophysics

The absorption maxima and extinction coefficients for the $La(2)^{3+}$ complexes are

30   found in Table I. The extinction coefficients were calculated assuming a formula for each complex of [La(2)]Cl₃. These data are consistent with complexation with the free ligand. There is the expected red shift in the ligand - * bands as previously reported for the $Tb(2)^{3+}$ and $Eu(2)^{3+}$ complexes. Prodi, L.; Maestri, M.; Ziessel, R.; Balzani, V., *Inorg. Chem,* 1991,

30, 3798. However, the extinction coefficients for the $Tb(2)^{3+}$ and $Eu(2)^{3+}$ complexes listed in Table I are 20% - 40% higher than previously reported. *Id.* This may be accounted for by working with a more pure sample. This hypothesis is substantiated by the questionably pure elemental analyses reported by Prodi, et al. for the $[Eu(2)]Cl_3$ and $[Tb(2)]Cl_3$ complexes. *Id.*

5　　　The emission maxima are listed in Table 1 and are consistent with the reported maxima for these lanthanides. Xu, Y.-Y.; Hemmilä, I.A. *Anal. Chim. Acta* 1992, *256*, 9. Two expected emission bands were not reported, this was due to interference by the first harmonic of the excitation wavelength. The relative quantum efficiencies, (La), were determined by the equation:

10
$$\frac{phi\ (Tb)}{Area(Tb)} = \left[\frac{Scale(Tb)}{Scale(La)}\right] \times \left[\frac{phi\ (La)}{Area(La)}\right]$$

where (Tb) is the reported quantum efficiency for $Tb(2)^{3+}$, 0.37 ± 0.1^{3}, area represents the area under the emission bands and is estimated by the weight of the paper

15　　within the emission bands, and Scale is the instrument scaling factor. Each weight used in the calculation was an average weight from four separate emission spectra. The relative quantum efficiencies are reported in Table I. Also included are the deviations which are based upon the reported deviation in the $Tb(2)^{3+}$ quantum efficiency and the standard deviations in each average weight. The largest portion of the listed quantum efficiencies was

20　　due to the reported 30% deviation in the quantum efficiency for $Tb(2)^{3+}$. Additionally, the quantum efficiency for the $Sm(2)^{3+}$ complex is expected to be higher than reported in Table I since the largest emission band for this complex was obscured by the second harmonic of the excitation wavelength. The quantum efficiency measurements will be repeated with a band-pass filter between the source and the sample to eliminate the second harmonic of the

25　　excitation wavelength.

Also included in Table I are the photophysical data for $Tb(1)^{3+}$ and $Eu(1)^{3+}$ as reported by Alpha et al. Alpha, B.; Ballardini. R.; Balzani, V.; Lehn, J.-M; Perathoner, S.; Sabbatini, N., *Photochem. Photobiol.* 1990, *52*, 299.

Table I. Photophysical Data

Complex	λ_{ex}(nm)	ε_{ex}(M^{-1}cm^{-1}) x 10^{-4}	λ_{em}(nm) [a]	ϕ_{rel} [c]
Eu(1)$^{3+}$ [e]	304	2.50	615 [f]	0.02 ± 0.006
Tb(1)$^{3+}$ [e]	304	2.90	542 [f]	0.03 ± 0.009
Tb(2)$^{3+}$	244 312	1.64 2.80	491 546 587 b	0.37 ± 0.1 [d]
Eu(2)$^{3+}$	244 312	1.85 3.02	584 596 620	0.033 ± 0.01
Sm(2)$^{3+}$	244 312	1.88 2.90	568 605 b	0.011 ± 0.003
Dy(2)$^{3+}$	244 312	1.75 2.95	481 575	0.020 ± 0.006

a. As measured with air equilibrated aqueous solutions that have an absorbance of 0.10 at 31 nm.

b. Expected La^{3+} emission maxima not measured due to excitation harmonic.

c. Quantum efficiencies determined relative to reported yield for Tb(2)$^{3+}$. All solutions an absorbance of 0.105 at the 320 nm excitation wavelength.

d. Prodi, L.; Maestri, M.; Ziessel, R.; Balzani, V., *Inorg. Chem,* 1991, 30, 3798.

e. Alpha, B.; Balzani, V.; Lehn, J.-M; Perathoner, S.; Sabbatini, N., *Angew. Chem. Int. Ed. Engl.* 1987, 26, 1266.

f. most intense emission band.

Electrochemistry

No oxidative redox processes were observed for the La(2)$^{3+}$ complexes within the 0.0 V to +2.5 V potential window. There were, however, several reductive redox processes within the 0.0 V to -2.3 V potential window. Three of the complexes have similar processes and cyclic voltammograms; Dy(2)$^{3+}$, Sm(2)$^{3+}$, and Tb(2)$^{3+}$. Figure 2 contains the cyclic voltammogram of Dy(2)$^{3+}$ which is representative of this group of complexes. These complexes have a group of three reductive waves between -1.0 V and -1.5 V. These waves correspond to one electron reductions for each of the bpy pendent arms of ligand 2. The final one electron reductive wave is being assigned as the second reduction of one bpy arm, the other second reductions are presumed to be just beyond the potential window. The cyclic voltammogram of Eu(2)$^{3+}$ is found in Figure 3 and shows a one electron reduction at -0.28 V and a set of three-one electron reductions between -1.7 V and -2.1 V. The first reduction is assigned as the Eu$^{III/II}$ couple based upon previously reported Eu$^{III/II}$ couple. Alpha, B.; Lehn, J.-M.; Methis, G., *Angew. Chem. Int. Ed. Engl.* 1987, 26, 266. The set of three reductions are assigned as the first reductions of each bpy arm. The set of bpy reductions for Eu(2)$^{3+}$ are shifted to more negative potentials as compared to those of Tb(2)$^{3+}$, Sm(2)$^{3+}$, and Dy(2)$^{3+}$; since it is more difficult to reduce a +2 complex than a +3 complex. Table II lists the electrochemical data for the La(2)$^{3+}$ complexes as well as the redox couple assignments.

Table II. Electrochemical Data [a]

Complex	La$^{3+/2+}$ [b]	bpy$_1$$^{0/-}$ [b]	bpy$_2$$^{0/-}$ [b]	bpy$_3$$^{0/-}$ [b]	bpy$_1$$^{-/2-}$ [b]
Eu(1)$^{3+}$	-0.56 [d]				
Tb(1)$^{3+}$					
Tb(2)$^{3+}$		-1.08 (80)	-1.28 (90)	-1.48 (90)	-2.05 (110)
Dy(2)$^{3+}$		-1.07 (90)	-1.26 (80)	-1.46 (80)	-2.04 (110)
Sm(2)$^{3+}$		-1.07 (60)	-1.27 (70)	-1.46 (60)	-2.02 (100)
Eu(2)$^{3+}$	-0.28 (110)	-1.59 (80)	-1.73 (90)	-1.89 (90)	-2.1 (E$_{pc}$) [b]

a. Values are E$_{1/2}$ in V vs. 3M Ag/AgCl and values in parentheses are ΔE in mV.
b. assignment of the redox process
c. an irreversible process with only the anodic peak present.
d. Alpha, B.; Lehn, J.-M.; Methis, G., *Angew. Chem. Int. Ed. Engl.* 1987, 26, 266.

ECL Measurements

Plots of the point-by-point background corrected ECL counts vs. potential curves for
the La(1)$^{3+}$ complexes are shown in Figure 4. These results show that both complexes have
similar potential responses. The onset of ECL for both complexes occurs at ca.
-1.4 V. Peak potentials are observed at ca. -2.8 V. However, the ECL vs. potential curve for
the Tb(1)$^{3+}$ complex shows a shoulder at ca. -1.7 V where this is not observed in the Eu(1)$^{3+}$
case. The shoulder, if real, is presumably due to ECL generated by the mediated
mechanism. The potential step-ECL decay curves for the La(1)$^{3+}$ complexes (Figure 5)
show a biphasic decay. A very rapid decay is observed after the potential was applied,
followed by a pseudo-plateau which starts to decay to baseline at ca. 8 s. This final decay is
presumed to be due to consumption of persulfate. ECL vs. La(1)$^{3+}$ concentration curves are
found in Figure 6. These data are based upon the potential step results. These data show a
linear response over the concentration range
(10000 nM) studied. A notable exception from the linear response is the 10000 nM Tb(1)$^{3+}$
data point. This lower than expected signal (47%) may be due to changes in the ECL
mechanism from the direct mechanism (reduced label reacting with reduced persulfate) to
the mediated (reduced persulfate oxidizing the label which is then reduced at the electrode)
mechanism which is expected at μM label concentrations. Detection limits for these
complexes have been estimated. An estimate of the system noise, 10% the background ECL,
was used to determine the detection limits. Listed in Table III are the peak potentials and
detection limits for these complexes.

Point-by-point background corrected ECL vs. potential curves for the Tb(2)$^{3+}$,
Sm(2)$^{3+}$, and Dy(2)$^{3+}$ complexes are shown in Figure 7. The curves are similar for these
complexes. The ECL commences at ca. -2.2 V and increases until the edge of the potential
window (-5 V) is reached. The peak potentials for these complexes are -5 V and are
presumably associated with the ligand reduction. A potential step-ECL decay curve for each
of these complexes is shown in Figure 8. The curves for the Tb(2)$^{3+}$ and Sm(2)$^{3+}$ complexes
show that the ECL is noisy but constant over the 1 s pulse width; however, the ECL of
Dy(2)$^{3+}$ decays over the pulse width. ECL vs. label concentration curves for Tb(2)$^{3+}$,
Sm(2)$^{3+}$, and Dy(2)$^{3+}$ are shown in Figure 9. The Dy(2)$^{3+}$ and Sm(2)$^{3+}$ complexes have a
linear response over the concentration range studied (100 nM to 10000 nM for Dy^{3+} and 250
nM to 10000 nM for Sm^{3+}). The response for the Tb(2)$^{3+}$ complex is linear between 5 nM

and 1000 nM; however, the 10000 nM data show a 90% drop-off from expected. The source of this large deviation from the expected is unknown. No ECL was observed for the $Eu(2)^{3+}$ complex over the concentration range studied (1 nM to 10000 nM). This has been attributed to the negative shift in the ligand reductions being large enough to put them outside the potential window. Table III lists the detection limits for the $La(2)^{3+}$ complexes as well as the peak potentials.

Table III. Aqueous ECL Data [a]

Complex	Pp (V) [b]	Detection Limits (nM)
$Eu(1)^{3+}$	-2.8	1000
$Tb(1)^{3+}$	-2.8	10
$Tb(2)^{3+}$	-5.0	5
$Sm(2)^{3+}$	-5.0	250
$Dy(2)^{3+}$	-5.0	100
$Eu(2)^{3+}$	c	> 10000

a. Measured on ORIGEN Analyzer #215 equipped with gold electrodes and blue sensitive PMT (R268 at 800 V) in 50 μM Persulfate buffer (pH 6).

b. ECL peak potentials measured with a potential ramp from 0 V to -5.0 V at 4800 mV/s.

c. No measurable ECL up to listed detection limit.

Discussion

The proposed mechanism by which ECL is generated by these lanthanide compounds is complex. This must be the case for $Tb(1)^{3+}$, $Tb(2)^{3+}$, $Sm(2)^{3+}$, and $Dy(2)^{3+}$ since the ECL stems from an f-f excited state. This excited state cannot be populated in a redox process analogous to Equation (3) since these complexes do not have a metal based reduction within the working potential window. However, there is a ligand based reduction within the potential window. Therefore, we are proposing that the light is obtained via Antenna ECL. The following steps summarize in Equations (4), (5) and (6) below the Antenna ECL mechanism.

(4) $La(L)^{3+} + e- \qquad La(L)^{2+}$

(5) $La(L)^{2+} + O_4- \qquad La(L*)^{3+} + SO_4{}^{2-}$

(6) $La(L*)^{3+} \qquad La*(L)^{3+}$
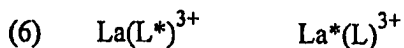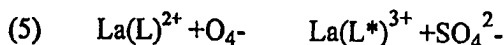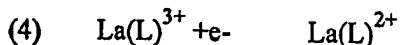
5   In Equation (4) the ligand (L) is reduced and following the homogeneous electron transfer, an intraligand excited state is formed, Equation (5), via electrochemical excitation (ECX). This excited state sensitizes the f-f excited state in an intramolecular energy transfer step, Equation (6), analogous to the mechanism by which f-f emission is observed in a fluorescence experiment. This mechanism is supported by the change in ECL peak potential

10   with a change in ligand between ligands 1 and 2. This new ECL mechanism is analogous to the mechanism by which the efficient lanthanide fluorescence is obtained with these complexes. That is the highly absorbing ligand bound to the lanthanide sensitizes the f-f excited state. The notable difference being the mechanism of ligand excitation. In the fluorescence a technique, ligand excitation occurs via absorption of a photon. Since a large

15   majority of biologically relevant species and the media absorb the light needed for ligand excitation fewer photons will produce the ligand excited state, and since many of these species will also emit there will be a high background signal. Therefore, this excitation mechanism can be a disadvantage for the use of lanthanides in immunoassays. However, fewer biologically relevant species and the media will emit via ECX. If ECX and Antenna

20   ECL are used to produce the lanthanide emission one would expect to have an assay with fewer interferences.

       A comparison of the quantum efficiencies and ECL efficiencies is useful. The ECL efficiency is defined as the moles of photons per 2 moles of electrons (2 electrons are needed to produce one reduced labels and one reduced persulfate). The quantum efficiency is

25   defined as the moles of photons per mole of excited states. These two equations are identical when all reduced labels react with all reduced persulfate to produce a label excited state. Therefore, a comparison of these two values can gauge the efficiency of the electron transfer reactions. However, in the aqueous ECL reactions the media is also being reduced. This prevents an exact determination of the ECL efficiencies. If one uses ECL detection limits in

30   place of ECL efficiencies, a trend analysis can be made which will eliminate interferences due to media reduction.

Dy(2)$^{3+}$, and Sm(2)$^{3+}$. However, the remaining complexes do not allow the trend for various reasons. The complex Tb(1)$^{3+}$ does not follow the trend since complexes of ligand 1 and complexes of ligand 2 are expected to have differences in electron transfer rates since the ligands have different reduction potentials (see Table II). The Eu^{3+} complexes cannot be

5   compared to the others due to some major differences. All of these differences are due to the presence of the Eu$^{III/II}$ couple prior to the ligand reductions. Different electron transfer rates are expected since the first ligand reduction is for Eu^{2+} complex instead of Eu^{3+} complex; therefore, the ligand reductions occur at more negative potentials than the other complexes with the same ligand. Also differences in excited state or in the mechanism of excited state formation are

10   expected. Since the ligand excited state is produced following Eu reduction, either a second oxidation must occur prior to Eu^{III} excited formation or a Eu^{II} excited state will be formed which is expected to have different photophysical properties such as emission efficiencies and maxima.

What is claimed is:

1. An electrochemiluminescent process for excitation of one or more luminophores through an antenna mechanism using an oxidative or reductive coreactant, wherein each luminophore is a complex with one or more ligands, comprising
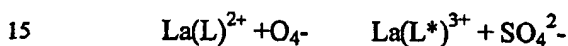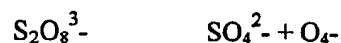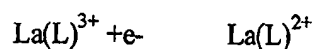
5   reacting one or more reduced luminophores with an oxidizing radical,

or reacting one or more oxidized luminophores with a reducing radical and generating a luminophore excited state and

measuring the electrochemiluminescent emissions.

2. The process of claim 1 wherein the luminophores are lanthanide chelates.

10   3. The process of claim 2 wherein the coreactant is a persulfate.

4. The process of claim 3 wherein the reactions in the process are as follows:

$La(L)^{3+} + e-$ $La(L)^{2+}$

$S_2O_8^{2-} + e-$ $S_2O_8^{3-}$

$S_2O_8^{3-}$ $SO_4^{2-} + O_4-$

15    $La(L)^{2+} + O_4-$ $La(L^*)^{3+} + SO_4^{2-}$

$La(L^*)^{3+}$ $La^*(L)^{3+}$

$La^*(L)^{3+}$ $La(L)^{3+} + hv$

wherein La represents lanthanide metal, L represents ligand, L represents a reduced ligand, L* represents a ligand excited state and La* represents a lanthanide excited state.

5. The process of claim 2 wherein the lanthanides are selected from the group consisting of Sm, Eu, Tb, and Dy.

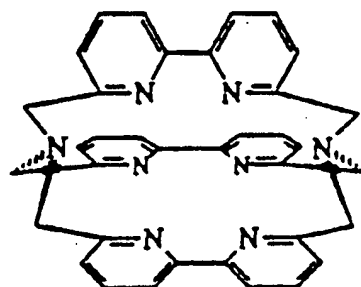6. The process of claim 2 wherein the ligands are selected from the group consisting of

2   1
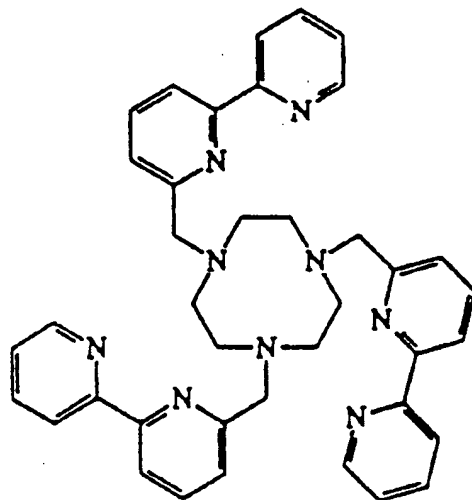
7. The method of claim 5 wherein the lanthanide chelates are complexes selected from the group consisting of $Sm(2)^{3+}$, $Eu(1)^{3+}$, $Tb(2)^{3+}$, $Tb(1)^{3+}$, and $Dy(2)^{3+}$.

8. An electrochemiluminescent process for excitation of one or more lanthanide chelates, each lanthanide chelate comprising a lanthanide complex with one or more ligands, through an antenna mechanism using an oxidative or reductive coreactant comprising

      reacting one or more reduced ligands with an oxidizing radical or reacting one or more oxidized ligands with a reducing radical and generating a ligand excited state; and

      measuring the energy transfer from the ligand excited state to the emissive state.

9. The process of claim 8 wherein two or more lanthanide chelates are employed and the electrochemiluminescent emissions are measured at different emission wavelengths or different electrode potentials.

10. The process of claim 8 wherein the coreactant is a persulfate.

11. In a system for electrochemiluminescent excitation and emissions measurement employing a luminophore and a coreactant, the improvement comprising employing a lanthanide chelate as the luminophore and a persulfate as the coreactant.

12. The system of claim 11 wherein two or more luminophores are employed and the emissions are measured at different emission wavelengths or different electrode potentials.

13. A method of simultaneously determining the presence of two or more different analytes which comprises

  contacting a sample suspected of containing one or more analytes with two or more different lanthanide chelates in the presence of a coreactant under conditions for measuring electrochemiluminescence,

  measuring electrochemiluminescence at different emission wavelengths or different electrode potentials,

  comparing the measurements to a standard.

14. The method of claim 13 wherein the coreactant is a persulfate.

15. A kit for simultaneously determining the presence of two or more different analytes which comprises

  two or more different lanthanide chelates in premeasured amounts;

  a coreactant in a premeasured amount; and

  a reference standard,

wherein the premeasured amounts are sufficient to perform a single sample measurement.

16. The kit of claim 15 wherein the coreactant is a persulfate.

17. The kit of claim 15 further comprising a means of generating electrochemiluminescence and a means of measuring electrochemiluminescence at different emission wavelengths or different electrode potentials.
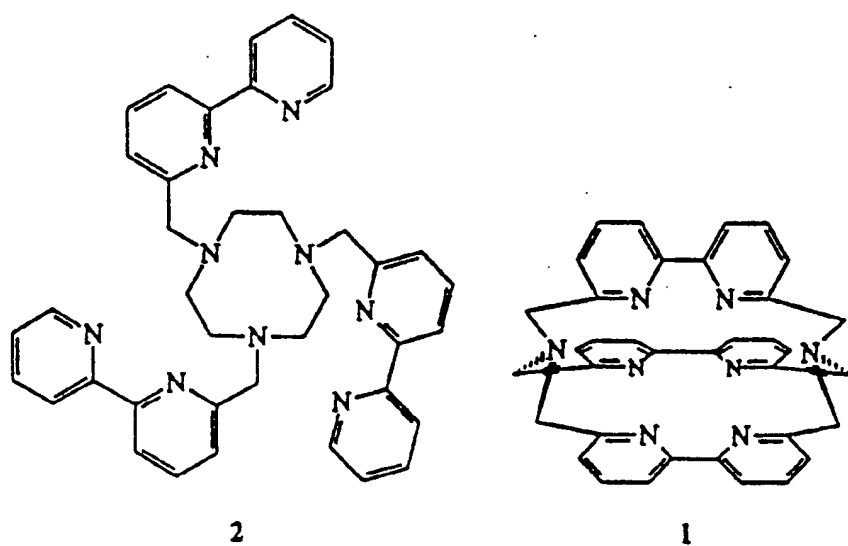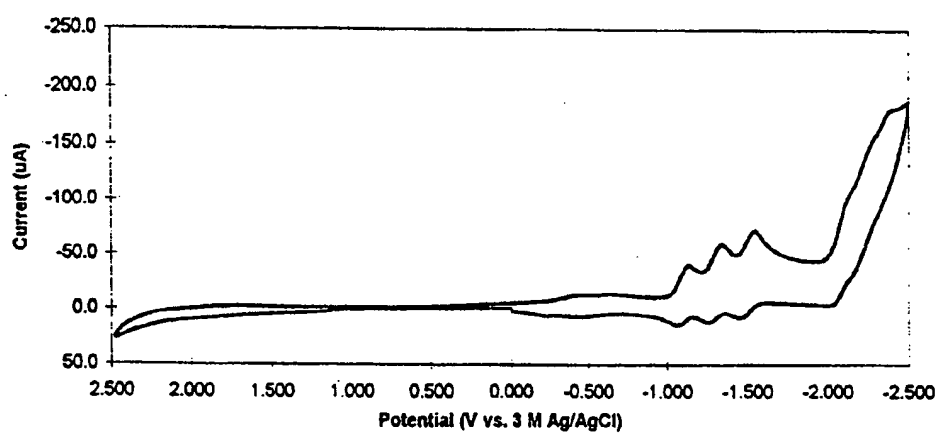
1/9

Figure 1. Ligand Structures

Figure 2.   Cyclic Voltammogram of Dy(2)$^{3+}$
Acetonitrile solution 0.1 M in TBAP and 5 mM in La^{3-}
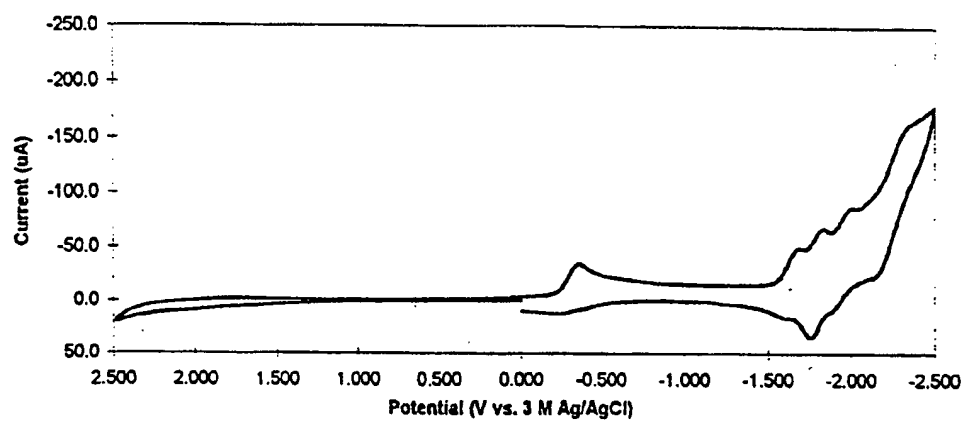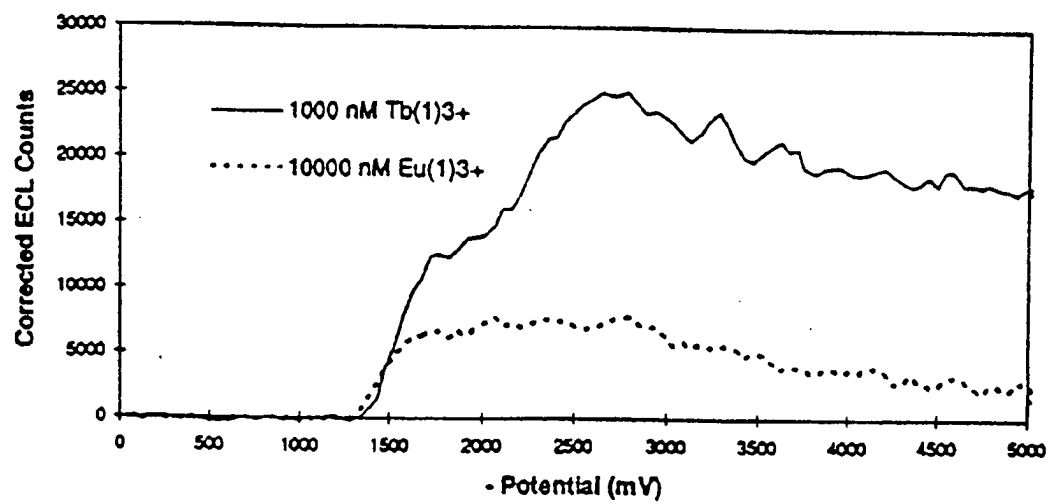Recorded on platinum electrodes in V vs. 3 M Ag/AgCl

Figure 3. Cyclic Voltammogram of Eu(2)$^{3+}$
Acetonitrile solution 0.1 M in TBAP and 5 mM in La^{3+}
Recorded on platinum electrodes in V vs. 3 M Ag/AgCl
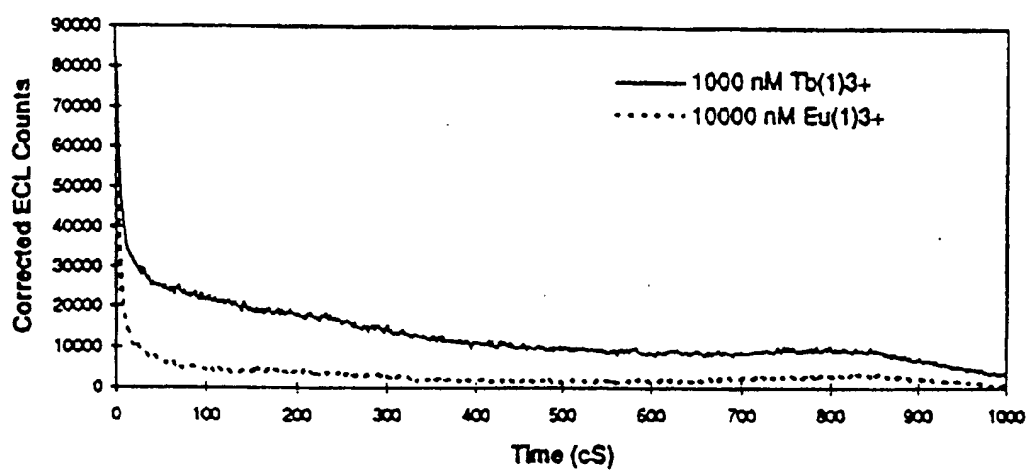
Figure 4. Aqueous La(1)$^{3+}$ Ramp ECL
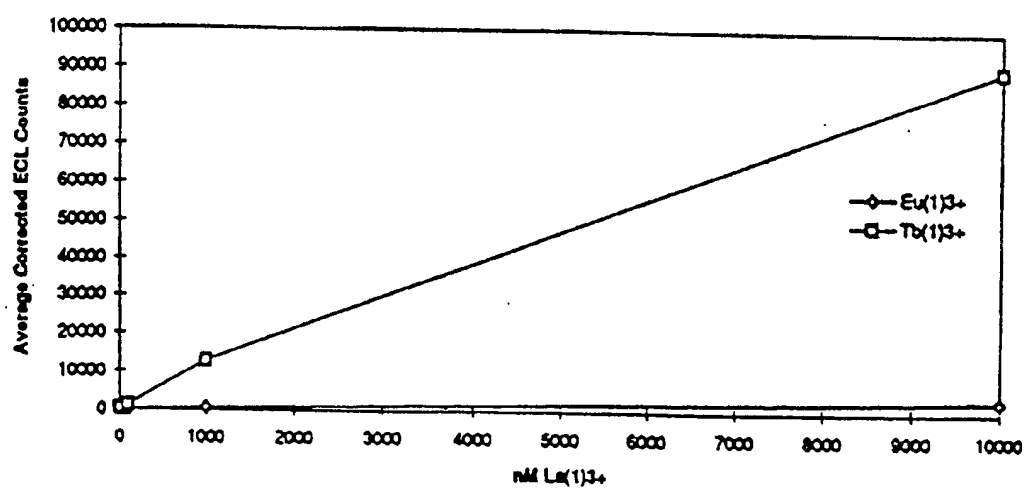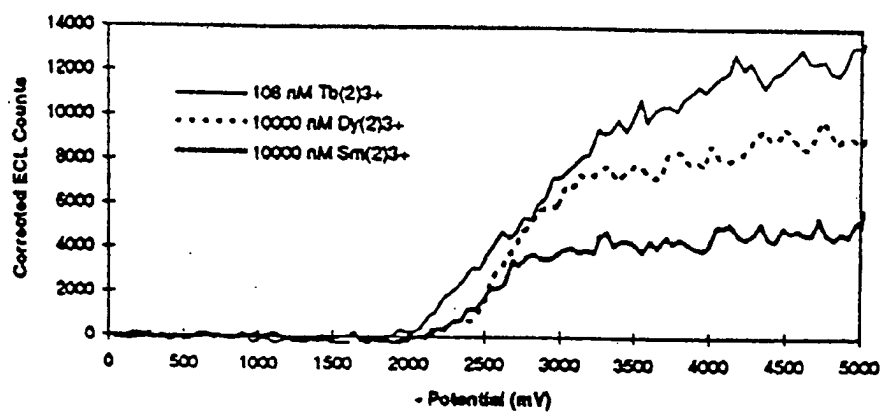
Figure 5. Aqueous La(1)$^{3+}$ ECL Step (-5 V)

Figure 6. Aqueous La(1)³⁺ Calibration Curve
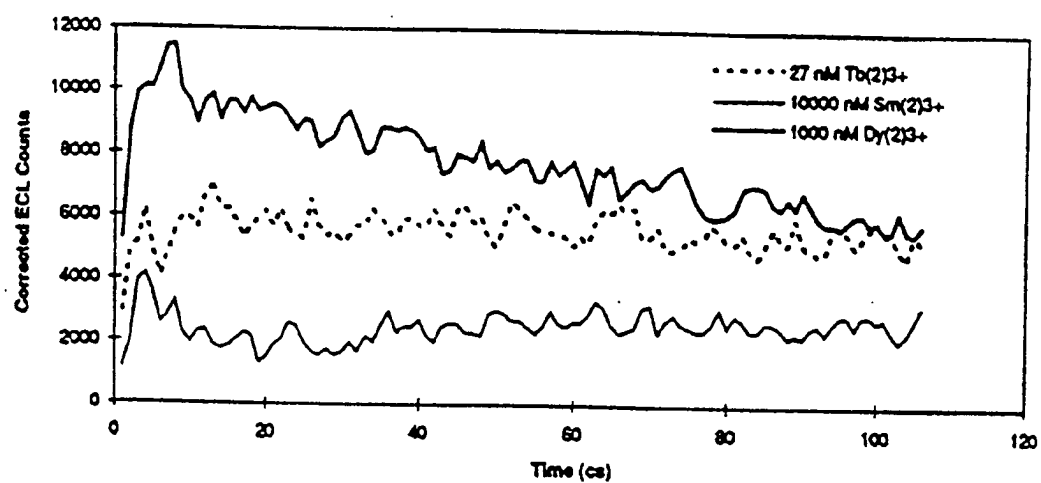
Figure 7. Aqueous La(2)$^{3+}$ Ramp ECL

Figure 8. Aqueous La(2)$^{3+}$ ECL Step (-5 V)

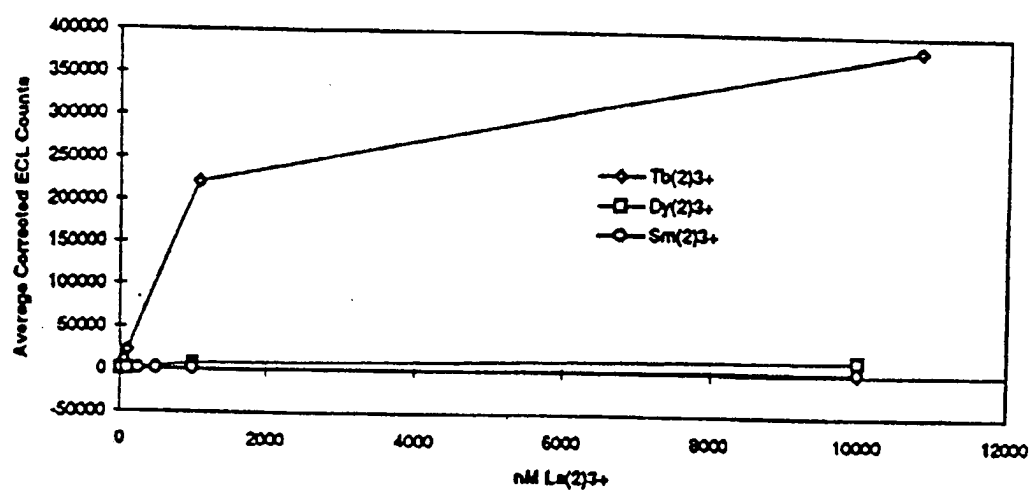Figure 9. Aqueous La(2)³⁺ Calibration Curve

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/536, 33/542; C09K 11/77; C07F 15/00
US CL :436/172, 536, 537, 805; 534/15; 252/521, 700

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/172, 536, 537, 805; 534/15; 252/521, 700

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched -

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, CAS ONLINE
search terms: electrochemiluminescence, Sm, Tb, Eu, Dy, lanthanide, electroluminescence

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	Kankare, J. et al. Cathodically induced time-resolved lanthanide(III) electroluminescence at stationary aluminium disc electrodes. Analytica Chimica Acta. 1992, Vol. 256, pages 17-28, especially Figures 7, 11 and 12.	1-5, 7-12 ------------ 6, 13-17
Y	US 5,308,754 A (J.J. KANKARE ET AL.) 03 May 1994, column 1, lines 53-62; column 2, lines 4-35; column 3, lines 13-68.	13-17
X ---- Y	US 5,310,687 A (A.J. BARD ET AL.) 10 May 1994, column 12, lines 30-49; column 17, line 41 to column 18, line 13; columns 20-22.	1 ------ 13-17

☒ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 SEPTEMBER 1996	10 OCT 1996

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARY E. CEPERLEY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YANG et al. Electrochemiluminescence: A New Diagnostic and Research Tool. Bio/Technology. 12 February 1994, Vol. 12, pages 193-194, especially page 194, first column, last paragraph and Table 1.	1
A	SABBATINI et al., Luminescent lanthanide complexes as photochemical supramolecular devices. Coordination Chemistry Review. 1993, Vol. 123, pages 201-228, especially pages 206 and 207, D. FLUOROIMMUNOASSAY.	13-17

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/09870

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s)1-12, drawn to a method for exciting luminophores.
Group II, claim(s) 13-17, drawn to a method for analyte tection and a kit therefor.

The inventions listed as Groups do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
The specific binding assay of Group II does not require that the electrochemiluminescent emission occur through an antenna mechanism as recited in Group I, nor does it require the use of the ligand of Group I.